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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, 5/10, A61K 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/34955</b> <b>(43) International Publication Date:</b> 7 November 1996 (07.11.96)
<b>(21) International Application Number:</b> PCT/US96/04902 <b>(22) International Filing Date:</b> 10 April 1996 (10.04.96)  <b>(30) Priority Data:</b> 08/434,201      3 May 1995 (03.05.95)      US  <b>(71) Applicant:</b> WARNER-LAMBERT COMPANY [US/US]; 201 Tabor Road, Morris Plains, NJ 07905 (US).  <b>(72) Inventors:</b> BARAGI, Vijaykumar, M.; 2838 Renfrew, Ann Arbor, MI 48105 (US). ROESSLER, Blake, J.; 3175 Dolph, Ann Arbor, MI 48103 (US).  <b>(74) Agents:</b> RYAN, M., Andrea; Warner-Lambert Company, 201 Tabor Road, Morris Plains, NJ 07950 (US) et al.		<b>(81) Designated States:</b> CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF TREATING CARTILAGINOUS DISEASES WITH GENETICALLY MODIFIED CHONDROCYTES  <b>(57) Abstract</b>  Described is a method of treating an arthritic condition comprising: subjecting a patient in need thereof to an effective amount of recombinant chondrocytes comprising articular chondrocytes recombinantly modified with a gene which has a therapeutic or prophylactic activity for an arthritic condition.		

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**METHOD OF TREATING CARTILAGINOUS DISEASES  
WITH GENETICALLY MODIFIED CHONDROCYTES**

**Technical Field**

5       The invention is concerned with the utilization of articular chondrocytes and the treatment of cartilaginous diseases. In particular, the invention pertains to chondrocytes that have been subjected to recombinant engineering.

**Background Art**

10       Chondrocyte-mediated cartilage degradation is a common end point in the pathogenesis of various human arthropathies including osteo- (OA) and rheumatoid arthritis (1-6). However, therapeutic strategies aimed at developing chondroprotective agents are faced with  
15   the challenge of targeting these agents to cartilage, an avascular tissue. Given the limitations of traditional methods of drug delivery, gene therapy offers a new approach for treating these diseases (7-11). Both in  
20   vitro and in vivo techniques using viral and non-viral vectors have been used to deliver lacZ, neo and Interleukin-1 Receptor Antagonist (IRAP) genes to synoviocytes/synovium (12). However, so far gene  
25   delivery techniques used have been unsuccessful in delivering genes to chondrocytes and /or articular cartilage. Interestingly, delivery of IRAP gene to synovium was effective in inhibiting many of the IL-1 effects, but had no significant effect on cartilage degradation (10-11). These results once again emphasize the need for delivery of therapeutic genes to cartilage.  
30   Knowing that chondrocyte-mediated cartilage degradation is a major pathway in the pathogenesis of various human

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arthropathies, it appears that the problem of drug delivery to cartilage still remains to be answered for treating these diseases.

Recently, it has been demonstrated that autologous chondrocytes, grown in tissue culture, introduced into a knee-cartilage defect, and sealed over with a periosteal flap, can lead over time to the growth of material resembling normal cartilage in human patients (13). This treatment was applied to more or less solitary lesions in an otherwise relatively normal joint. Cartilage degradation in an arthritic joint is an end result of catabolic processes outstripping anabolic processes (5-6); therefore, it is to be seen whether chondrocytes transplantation alone would be sufficient to heal arthritic cartilage. Nevertheless, chondrocytes transplantation offers another approach for treating cartilage. In the present study, combining chondrocytes transplantation and adenovirus-mediated delivery of IRAP gene, we have demonstrated that cartilage can be protected from IL-1 (Interleukin-1) - induced matrix degradation.

IL-1, a cytokine present in arthritic joint fluid (14, 15 ) and known to induce cartilage degradation (15,16), generate and maintain synovial inflammation (17), up regulate matrix metalloproteinase (18) and prostaglandin production (19), is implicated in the pathophysiology of arthritic joints. IRAP is considered to be a potentially therapeutic protein because of its ability to counteract many of the effects of IL-1. There have been a number of *in vitro* and some *in vivo* studies demonstrating the beneficial effects of IRAP, and an extensive review of this literature has been published (20).

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One of the issues with chondrocytes is that they are not readily accessible in the body in that they constitute a very small fraction of the cartilage matrix. In particular, chondrocytes are cells that have  
5 no blood supply and rely upon materials to be diffused to them. This, therefore, makes them quite different from synovial cells which, in fact, do have a blood supply. Further, chondrocytes are embedded in the cartilage matrix whereas synovial cells are not. Thus  
10 drug delivery to chondrocytes is constrained by the matrix materials. Furthermore, the chondrocytes have a low replication rate.

U.S. Patent No. 5,399,346 is a general discussion with respect to gene therapy. Modification  
15 of chondrocytes are not suggested by the reference.

U.S. Patent No. 5,310,759 teaches methods of protecting and preserving connective and support tissues. The patent indicates that a pharmaceutically acceptable prostaglandin  $E_1$  and cyclic adenosine  
20 monophosphate agonists and inducers can selectively inhibit collagenase gene expression in human and animal (mammal) cells. The patent does not pertain to recombinantly modified chondrocytes.

PCT Publication W092/11359 published July 9,  
25 1992 teaches truncated interleukin-1 receptor gene for the treatment of arthritis. The specification indicates that a major source of IL-1 in the joint is the synovium. The specification does not teach the genetic modification of recombinant chondrocytes by gene  
30 therapy.

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PCT Specification WO94/01139 published January 20, 1994 pertains to targeting somatic gene therapy to joints. The invention is particularly concerned with a transfected synovial gene. The invention is not  
5 concerned with a recombinant or genetic modification of chondrocytes and the treatment of arthritic diseases with such chondrocytes.

German Application 4,219,626 published December 23, 1993 teaches incorporating a therapeutic  
10 gene via a vector into body cells *in vivo* and *in vitro* for subsequent expression and secretion of active protein, particularly for treating the diseases of spine and nerves. The reference does not specifically teach recombinant articular chondrocytes for treatment of  
15 cartilaginous diseases.

Intra articular expression of biologically active IL-1 receptor antagonist protein by *ex vivo* gene transfer is discussed in the paper by Bandara, Jean et al. *PNAS USA* (1993), 90 (22,), 10764-8. The document  
20 discloses the use of retroviral vectors to transduce synovial cells. In another paper entitled *Gene Transfer To Synoviocytes: Prospects For Gene Treatment of Arthritis* By Bandara, Jean et al. *DNA Cell Biol.* (1992), 11(3), 227-31 the use of synovium materials is  
25 discussed. Retroviruses are likewise disclosed. Recombinantly engineered chondrocytes are not discussed.

#### Summary of the Invention

Disclosed is a method to genetically modify chondrocytes using gene therapy. The method involves  
30 genetic alteration of articular chondrocytes so as to

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contain at least one gene encoding for a protein with therapeutic or prophylactic activity for an arthritic condition.

Also disclosed are transfected chondrocytes comprising articular chondrocytes genetically modified to contain at least one gene encoding for a protein with therapeutic or prophylactic activity for an arthritic condition.

Also disclosed is a method treating an arthritic condition comprising the steps of: subjecting a patient, in need thereof, to an effective amount of recombinant chondrocytes comprising mortal articular chondrocytes recombinantly modified with at least one gene which has therapeutic or prophylactic activity for an arthritic condition.

Also disclosed is a method of delivering a gene to cartilage comprising the steps of providing chondrocytes modified with at least one gene encoding for a protein which has therapeutic or prophylactic activity for an arthritic condition, and delivering the modified chondrocytes to a mammal having a diseased cartilaginous state.

#### Brief Description of the Drawings

FIGURE 1 is a photomicrograph reflecting the expression of transgenic *LacZ* by human chondrocytes. Cells expressing the transgene have a blue staining nuclei (A) and nuclei of control cells are not stained (data not shown). Immunohistochemical staining of

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transduced cells for expression of type II (B) and type I (C) collagen are shown.

FIGURE 2 is a photomicrograph of expression of transgenic *LacZ* by human chondrocytes transplanted onto cartilage organ cultures. Photograph 2A shows cells transduced with Ad.RSVnt*LacZ* vector two days after transplantation onto cartilage organ cultures.

FIGURE 2B is the same as Figure 2A except after eight days after transplantation.

FIGURE 2C shows cartilage with cells subjected to mock transfection which did not stain.

FIGURE 2D shows a photomicrograph of chondrocytes transduced with Ad.CMVL*LacZ* vector and transplanted onto the surface of intact cartilage. The cells exhibit cytoplasmic staining.

FIGURE 2E is a photomicrograph showing control cartilage with mock transfected cells which show no staining.

FIGURE 2F is a photomicrograph of scanning electron micrograph of cartilage showing transplanted chondrocytes embedded in territorial matrix.

FIGURE 2G is similar to Figure 2F but shows cells at a higher magnification.

FIGURE 3 is a graph demonstrating expression of human IL-1ra by transduced chondrocytes transplanted onto cartilage organ cultures.



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FIGURE 4 is a graph showing that transplantation of transduced chondrocytes overexpressing human IL-1ra protects cartilage organ culture from IL-1 mediated proteoglycan degradation.

5                   FIGURE 5 shows restriction maps for the generation of Ad.RSVntLacZ.

FIGURE 6 shows the restriction maps for the generation of Ad.CMVLacZ.

10                   FIGURE 7 shows the restriction maps for the generation of Ad.RSVhIL-1ra.

#### Description of Preferred Embodiments

15                   The present invention is concerned with the use of genetically modified chondrocytes. The chondrocytes are generally obtained from a patient, subjected to genetic manipulation and then inserted back into the very same patient. This is called the "ex vivo" technique. Alternatively, chondrocytes can be obtained from another source, subjected to genetic manipulation and then introduced into desired patients  
20                   where there is not a significant immunological response by the host. This is also called the "ex vivo" technique. However, in the first instance, autologous chondrocytes are used, whereas in the second example, allogenic chondrocytes are used.

25                   In a preferred embodiment, mortal chondrocytes are used. By "mortal" chondrocytes is meant chondrocytes that are not immortal but have senescence.

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Reference is made to genetic modification or genetic engineering or recombinant modification. By this is meant that there is an insertion of a gene into a DNA vector, often a plasmid to form a new DNA molecule that can be perpetuated in a host. This is also called recombinant DNA technology, genetic engineering, gene transplantation. Other techniques for modifying cells are described in more detail below.

When utilizing the genetic modification techniques for the present invention, it can be stated that one inserts the gene that encodes for one particular protein into a viral vector. That viral vector is then transfected into a viral packaging cell line for the production of a viral particle that is capable of expressing the gene. Thereafter, since one is utilizing in this instance a viral particle, it can be said that one infects the cells of a mammalian host using the viral particle obtained from the viral packaging cell line.

In the application, reference is made to "transduction" which is the transfer of non-viral DNA by a virus to a cell.

In the specification, reference is made to "transfection" which is the transfer of DNA to a eukaryotic cell.

The technique for obtaining chondrocytes is well known in the art. It is likewise well known in the art as to how to subject some types of cells to genetic manipulation to insert appropriate genes which produce proteins and the like. In general, inserting DNA

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(deoxyribonucleic acid) into cells is a well known technique. See *Molecular Biotechnology: Principles and Application of Recombinant DNA* by B.R. Ghik et al. (1994) American Society For Microbiology (Washington, D.C.). The introduction of DNA can be done either transiently or permanently into cells. The methods for introducing DNA into cells include calcium phosphate coprecipitation, cationic liposome or lipofection, electroporation, DEAE-Dextran, receptor mediated endocytosis, particle mediated gene transfer, or for some cell types, naked DNA can be utilized. The appropriate genes can be introduced into any of the well known viral vectors preferably adenovirus, adeno-associated virus, retrovirus, alpha virus and herpes virus.

In addition, one may insert antisense or ribozyme expression vectors for controlling gene expression by cells. See *Molecular Biotechnology*, supra, and ribozyme patents listed, infra.

The therapies, with which Applicant is concerned, are to treat cartilaginous diseases, especially chondroprotection. In particular, Applicant is interested in treating or controlling osteoarthritis, rheumatoid arthritis, metabolic bone disorders, infectious arthritis such as septic arthritic disease, inflammatory arthritic syndromes, e. g., ankylosing spondylitis and/or sports injuries which involve cartilage or arthritic symptoms. In particular, Applicant is desirous of introducing one or more genes into a particular cell and to deliver multiple genes to the site of affliction. A first gene would be to cease or prevent the degradation of the ailment. The second

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gene would be used to treat or improve the ailment being treated. Along these lines, the classes of potential anti arthritic proteins that may be introduced together or alone into cells are cited in the table below.

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CLASSES OF POTENTIALLY ANTI-ARTHRITIC AGENTS

	<u>Class</u>	<u>Example</u>	<u>Rationale</u>	<u>Reference</u>
5	Cytokines excluding IL-1	IRAP	Antagonizes IL-1	A
		IL-4	Downregulates IL-1, TNF- $\alpha$ induces IRAP	C,D,E
		IL-10	Immuno-Suppressive, Induces IRAP	B,F
10		Ribozymes	Reduce cytokine mRNA levels	
15	Anticytokines	Soluble receptors for IL-1, TNF- $\alpha$ etc.	Antagonize their cognate cytokine	G,H
	Antiadhesion Molecules	Soluble ICAM-1	Inhibit cell-cell, cell-matrix interaction	I J
		Soluble CD44		
20	Anti-oxidants	Superoxide dismutase; NO synthase blockers	Prevents effects of free radicals	K,L
25	Cartilage growth factors	IGF-1	Promote	M
		TGF- $\beta$	Cartilage Repair	
30	Matrix Meta- lloproteinase Inhibitors	TIMP-1*, TIMP-2	Inhibit cartilage degradation mediated by Matrix Metallo- proteinase (MMP)	N
		Ribozymes	Reduce MMP mRNA levels	

\*TIMP = Tissue Inhibitor of Metalloproteinase

This Table is illustrative, not exhaustive.

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References

- A. Arend, W.P.: Interleukin-1 receptor antagonist. A new member of the interleukin-1 family. J. Clin. Invest. 88: 1445-1451, 1991.
- 5 B. DeWaal, M.R., Yssel, H., Roncarolo, M.G., Spits, H., DeVries, J.E.: Interleukin-10. Curr. Opin. Immunol. 4:314-320, 1992.
- C. Karlsson, S., Correll, P.H., Xu, L.: Gene transfer and bone marrow transplantation with special reference to Gaucher's disease. Bone Marrow Transplant, 10 11(suppl.1):124-127, 1993.
- D. Essner, R., Rhoades, K., McBride, W.H. Morton, D.L., Economu, J.S.: IL-4 downregulates IL-1 and TNF gene expression in human monocytes. J. Immunol. 142:3857-15 3861, 1989.
- E. Vannier, E., Miller, L.D., Dinarello, C.A.: Coordinated anti-inflammatory effects of interleukin-4: interleukin 4 suppresses interleukin 1 production but, upregulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc. Natl. Acad. Sci. USA 89: 20 4076-4080, 1992.
- F. Howard, M., O'Garra, A.: Biological properties of interleukin-10. Immunol. Today 13:198-200, 1992.
- G. Fanslow, W.C., Sims, J.E., Sassenfeld, H., 25 Morrissey, P.J., Grillis, S., Dower, S.K., Widner, M.B.:

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Regulation of alloreactivity *in vivo* by a soluble form of the interleukin-1 receptor. *Science* 248:739-742, 1990.

5 H. Fernandez-Botran, R.: Soluble cytokine receptors: their role in immunoregulation. *FASEB J.* 5:2567-2574, 1991.

10 I. Iigo, Y., Takashi, T., Tamatani, T., Miyasaka, M., Higashida, T., Yagita, H., Okumura, K., Tsukada, W.: ICAM-1-dependent pathway is critically involved in the pathogenesis of adjuvant arthritis in rats. *J. Immunol.* 147:4167-4171, 1991.

15 J. Galea-Lauri, J., Wilkinson, J.M., Evans, C.H.: Characterization of monoclonal antibodies against CD44: Evidence of a role for CD44 in modulating synovial metabolism. *Mol. Immunol.* In Press, 1993.

K. Skaleric, U., Allen, J.B., Smith, P.D., Mergenhagen, S.W., Wahl, S.M.: Inhibitors of reactive oxygen intermediates suppress bacterial cell wall-induced arthritis. *J. Immunol.* 147:2559-2564, 1991.

20 L. Stefanovic-Racic, M., Stadler, J., Evans, C.H.: Nitric oxide and arthritis. *Arthritis Rheum.* In Press, 1993.

25 M. Morales, T.I., Hascall, V.C.: Factors involved in the regulation of proteoglycan metabolism in articular cartilage. *Arthritis Rheum.* 32:1197-1201, 1989.

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N. Woessner, J.F. Jr., Matrix Metalloproteinases and Their Inhibitors In Connective Tissue Remodeling. FASEB.J. (1991): 5(8): 2145-54.

5       Of the above-identified numerated classes, the most preferred is to insert the gene for IRAP into the cell. This gene would thereby bind IL-1 which would prevent further inflammation in an arthritic environment.

10       Ribozymes and methods for their preparation have been disclosed in U.S. Patent Nos. 4,987,071; 5,037,746; 5,116,742; 5,093,246 and 5,180,818, which are hereby incorporated by reference.

15       The delivery method for the recombinant chondrocyte can be either by *ex vivo* or *in vivo* delivery. See Molecular Biotechnology, supra, pp.402-415.

20       For *in vivo* delivery, a suitable viral or non-viral delivery system is used to administer the desirable gene to the patient. This administration may be intravenous or intraarticular. Another formulation could be, for example, using cationic liposomes (Philip R. et al. J. Biol.Chem., 268: 16087-16090 (1993)) where from 10  $\mu$ g to 10 mg of a vector expressing the desired gene is delivered.

25       The usual dose would vary with each disease and therapeutic gene used to treat the disease. However, it is estimated that transplantation of  $1-5 \times 10^8$  cells/knee joint would produce requisite therapeutic protection.



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Listed below are preferred embodiments wherein all parts are parts by weight and all temperatures are degrees centigrade unless otherwise indicated.

Examples

5     Cartilage Organ and Chondrocytes Cultures

          The articular cartilage used in this study was obtained from osteoarthritis patients undergoing total knee or hip replacement surgery. The ages of the patients ranged from 50 to 70 years. To establish  
10     cartilage organ cultures, uniform cartilage slices measuring approximately 11 mm by 11 mm were dissected from the underlying bone using a #20 scalpel blade and placed in isotonic saline. The cartilage slices were washed in Gey's balanced salt solution and placed (one  
15     piece/well) in Falcon 24 well flat-bottom tissue culture plates (Becton Dickinson, Lincoln Park, NJ). The cartilage organ cultures were maintained in complete Ham's F-12 (supplemented with 10% fetal calf serum 6.5 mg /ml Hepes, 58.5 µg /ml glutamine, 200 µg/ml MgSO<sub>4</sub>,  
20     100 units/ml Penicillin G sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B) at 37° C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

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The chondrocytes needed for the study were isolated from the cartilage pieces separated from the underlying bone. The cartilage slices were minced finely and then digested sequentially with 0.2 % testicular hyaluronidase (5 minutes at 37°C), 0.2% trypsin (30 minutes at 37°C), and 0.2 % collagenase (1-2 hours at 37°C). The resulting solutions were strained through sterile nylon mesh and centrifuged at 1,200 x g for 10 minutes. The cell pellets were washed twice with Ham's F-12 medium, resuspended, combined and centrifuged at 1,000 x g for 10 minutes. The resulting pellet was suspended in complete F-12 medium (described above). This cell suspension was used to establish cell cultures in 25-cm<sup>2</sup> falcon flasks at a density of 1-2 x 10<sup>6</sup> cells /flask. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The chondrocyte used in this study were maintained as monolayer cultures for no more than 2 passages, in order to maintain the differentiated chondrocyte phenotype.

#### Recombinant Adenoviral Vectors

The adenoviral vectors used in this study are based on an adenoviral (serotype 5) genomic backbone deleted of sequences spanning E1A and E1B and a portion

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of E3 region (Restriction Map shown in Figures 5-7). This impairs the ability of this virus to replicate or transform non-permissive cells (21). Gene transcription was driven by the early enhancer/promoter of the cytomegalovirus (CMV) in the Ad.CMVlacZ vector, and 5 Rous sarcoma virus (RSV) long terminal repeat (LTR) in Ad.RSVntlacZ and Ad.RSVhIL-1ra vectors. An SV40 polyadenylation sequence was cloned downstream from each of these reporters (9-10). The vector Ad.RSVntlacZ 10 contains a nuclear targeting (designated as nt) epitope linked to the lacZ gene, and as result, the cells transduced with this gene can be identified by the presence of blue nuclei after reaction with the chromogenic substrate X-gal (as described below).

15 Transduction of Chondrocytes

Primary human chondrocyte cultures were treated with trypsin (Worthington Biochemicals Corp. Freehold, N.J.) and seeded in 6-well tissue culture plates at a density of  $0.5-1.0 \times 10^6$  cells/ well. After 20 the cells were 70% confluent, they were washed two times with serum free Dulbecco's Modified Eagle Media (DMEM), infected with Ad.RSVntlacZ (titer =  $10^{10}$  plaque forming units (pfu)/ml); diluted 1:100 in serum free DMEM) at an approximate multiplicity of infection (MOI) of  $10^3$  25 pfu/cell, incubated at  $37^{\circ}\text{C}$  for two hours. Cells were

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then washed two times in fresh DMEM. DMEM containing 10% fetal calf serum (FCS), antibiotics and amphotericin B was added to the cultures and the cells were allowed to grow for 24 hours at 37°C. Mock infected cells were maintained in parallel.

#### Chondrocyte Transplantation

Transduced allogeneic chondrocyte were treated with trypsin, suspended in 1-3 ml of complete Ham's F-12 medium and added slowly onto the articular surface of cartilage organ cultures or intact cartilage (with the underlying subchondral bone) selected for transplantation experiments. To examine optimal conditions for adherence of cells to cartilage, cartilage pieces were pretreated with 10-20% serum (Fetal calf, Life Technologies Inc., Grand Island, N.Y.), 0.5 % gelatin (Sigma, St Louis, Mo.) or 3.5  $\mu\text{g}/\text{cm}^2$  (surface area) Cell-Tak® (Collaborative Products, Bedford, Ma.). However, in most of the experiments, complete F-12 (containing 10% serum) used for maintaining the cartilage organ cultures was sufficient for adherence of cells to cartilage. Approximately  $0.5-1.0 \times 10^6$  cells were added to each cartilage piece and the cultures incubated for 48 hours in complete F-12 medium at 37 °C. In some of the experiments, to prevent

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cells from settling onto the plastic, the bottom surface of the tissue culture plates were treated with silicon and cartilage slices placed into these wells before the addition of chondrocyte.

5     In Situ Staining for  $\beta$ -galactosidase Transgene Expression

Chondrocytes were washed in phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in 100 mM PIPES pH 6.9 for 30 min. Cells were washed for 30  
10    minutes in PBS/2 mM  $MgCl_2$  and reacted in 5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4Fe(CN)_6$ , 2 mM  $MgCl_2$  0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal ) in PBS for 4 hours at 37°C (22). Cells were then washed three times with PBS, post-fixed  
15    for 2 hours in 2% paraformaldehyde/1% glutaraldehyde, counterstained with neutral red and photographed using light microscopy. A similar protocol was used for staining cartilage organ cultures. Briefly, twenty-four hours post-infection with adenoviral vectors,  
20    chondrocyte were treated with trypsin and added to cartilage organ cultures. At various times (1-8 days) post-transplantation, cartilage slices were fixed and reacted with X-gal. Both the fixing and X-gal staining procedures for the cartilage pieces were done over a

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period of 24-48 hours. Parallel samples were treated with trypsin and the recovered cells plated onto plastic dishes. Twenty-four hours after plating, cells were fixed and reacted with X-gal and photographed.

## 5 Immunohistochemistry

Duplicate samples of transduced cells were analyzed for the co-expression of ntlacZ and type II or type I collagen using immunohistochemical staining. Cells were fixed in cold methanol, then reacted with X-  
10 gal for 2 hours. Cells were washed twice in PBS then incubated for 1 hour with PBS/10% FCS containing monoclonal murine anti-human type II or I collagen (1:100) at 37°C. (Chemicon International, Temecula, Ca.). Cells were washed twice in PBS then incubated with  
15 goat anti-murine IgG conjugated to biotin (Histostain, Zymed Laboratories, Inc., San Francisco, Ca.). Cells were then reacted with Streptavidin-horseradish peroxidase followed by aminoethylcarbazole and examined by light microscopy.

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Transmission Electron Microscopy

Cartilage slices used for transplantation experiments were examined by scanning electron microscopy (SEM) to identify integration of transduced  
5 cells into the articular surface. Discs for SEM were trimmed to a width of 1 mm and fixed in 4% paraformaldehyde in PBS followed by 1% glutaraldehyde in 0.5 M cacodylate (Fluka Chemika, Switzerland) buffer. Discs were dehydrated in graded alcohols and coated with  
10 gold, then imaged using a ISC DS-130 scanning electron microscope.

Effects of Interleukin-1 on Cartilage Degradation and Transgene Expression

Twenty-four hours post-infection, the  
15 chondrocyte were treated with trypsin and plated onto human OA cartilage organ cultures and the cultures incubated in complete F-12 medium at 37°C. Forty-eight hours post-transplantation of chondrocyte, cartilage organ cultures were rinsed with 1 mL F-12 Nutrient  
20 Mixture (without serum) in order to remove residual FCS. Each treatment group was done in triplicate. The controls received 1 ml F-12 medium + 10 µl PBS with 0.1% BSA (bovine serum albumin) and IL-1 treatment group

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received 1ml F-12 medium + 10  $\mu$ L IL-1 $\alpha$  (500 Units).  
Cartilage organ cultures were incubated for 10 days.  
Every 48 or 72 hours, conditioned medium was replaced  
with fresh medium and IL-1 or PBS/BSA were added. All  
5 media changes were collected. At the end of the  
experiment, the cartilage pieces were subjected to  
papain digestion. Cartilage pieces were digested in 24  
well plates by adding 50  $\mu$ L papain in 1.0 mL cysteine  
buffer (0.1M phosphate buffer, pH 7.0 containing 0.01M  
10 L-cysteine HCl and 0.05M EDTA) and incubating at 56<sup>0</sup>C  
until the cartilage pieces were dissolved completely.  
Effect of IL-1 on proteoglycan degradation was  
determined by analyzing the cartilage digests and media  
samples collected for chondroitin sulfate content, using  
15 the modified 1,9-Dimethylmethylene Blue (DMB) assay  
(23). The conditioned media were also analyzed by ELISA  
(R & D Systems, Minneapolis, MN) for the presence of  
endogenous and transgenic human IL-1ra.

## Results

### 20 Transduction of Human Chondrocyte

To determine if human chondrocyte in culture  
are susceptible to infection and transduction by  
adenoviral vectors, lacZ was used as a marker gene. Two  
recombinant human serotype 5 (sub360 derivative)



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adenovirus vectors, Ad.RSVntlacZ and Ad.CMVlacZ were used to deliver the marker gene. The vector Ad.RSVntlacZ contained a nuclear targeting epitope linked to the lacZ gene, thus transduced cells could be identified by the presence of blue nuclei after reaction with the chromogenic substrate X-gal. Greater than 90% of the infected cells exhibited blue nuclei, indicating expression of the *E. coli lacZ* gene mediated by adenoviral gene transfer (Fig 1 A ). Similar results were obtained with cells infected with the Ad.CMVlacZ vector; the cells exhibited blue cytoplasmic staining (data not shown). Control cells (mock infected) exhibited no blue staining, indicating that the expression of  $\beta$ -galactosidase activity was specific for the transgene and did not represent endogenous enzyme activity (not shown). No abnormalities in cell shape or evidence of cell death were observed in the infected cells.

Furthermore, there was no evidence of morphological alterations and most cells (>70 %) expressed type II collagen, characteristic of chondrocyte phenotype (Fig 1B). However, a small percentage of cells did express type I collagen (Fig 1C). Thus, these experiments demonstrate that human chondrocyte in monolayer culture are readily infected

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with recombinant adenovirus and that adenovirus efficiently mediates transduction (gene transfer) and transgenic protein expression in vitro. Furthermore, transduced cells maintain their characteristic  
5 chondrocyte phenotype.

#### Transplantation of Transduced Chondrocytes

Transplantation of chondrocytes transduced with the *LacZ* and IRAP genes were undertaken as a means to transfer genes to articular cartilage. Twenty-four  
10 hours post-infection, chondrocyte were transplanted onto the surface of cartilage organ cultures. Examination of cartilage cultures for the presence of *lacZ* transduced cells at various times (1-8 days) post-transplantation showed no evidence of a decline in number or intensity  
15 of blue staining cells on the surface of cartilage cultures, indicating that *lacZ* expression remained about the same during the 8-day period (Fig 2 A, B). Cartilage samples with mock-transfected cells showed no evidence of cells with blue staining nuclei (Fig 2C),  
20 indicating that the presence of blue staining nuclei on the articular surface was specific for the Ad.RSVntlacZ transduced chondrocyte. Examination of intact cartilage with the underlying bone also revealed the presence of blue staining cells on the articular surface (Fig 2 D).

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Again cartilage samples with mock-transfected cells were negative for blue staining cells (Fig 2E). Thus, it is was evident that transduced chondrocyte would adhere to the surface of human OA cartilage, remain viable, and  
5 continue to express transgenic protein.

#### Electron Microscopy

Examination of the OA cartilage discs by SEM revealed that transduced chondrocytes were visible along the articular surface of seeded discs (Fig 2 F), but  
10 were not found on unseeded control discs (data not shown). Fig 2F shows transplanted chondrocyte on two pieces of cartilage that are embedded within the territorial matrix. Fig 2G is a higher magnification of cells adhered to cartilage. The cells display an  
15 irregular surface as well as interconnecting cytoplasmic processes, characteristics of viable, metabolically active cells. Bundles of collagen fibers are found surrounding each cell. These results indicate that transduced chondrocytes can adhere and integrate into  
20 the surface of OA cartilage.

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Transduction of Chondrocyte by IRAP Gene

The purpose of these experiments was two-fold. First, to determine if chondrocytes transduced with a recombinant adenovirus containing the cDNA for a biologically active cytokine antagonist also adhere to the surface of cartilage slices. Second, to determine if the transduced chondrocytes present on the surface of cartilage slices appropriately process and secrete a biologically active human cytokine antagonist. Cartilage organ cultures containing chondrocytes transduced with Ad.RSVIL-1ra expressed and secreted on an average 1  $\mu$ g/ml of human IRAP protein over a 10-day period; whereas, cartilage cultures containing chondrocyte transduced with Ad.RSVntlacZ produced on an average 100 pg/ml of IRAP (ANOVA:  $P < 0.0001$ ,  $F = 56.7$ ,  $N = 16$ ). Identical groups of cartilage stimulated with recombinant human IL-1 $\alpha$  (500 units/ml) showed no statistically significant increase in the expression of human IL-1ra. Thus, human chondrocytes transduced with cDNA for a soluble human protein can appropriately express, process and secrete an immunoreactive transgenic protein. Furthermore, the expression of this protein is independent of endogenous regulation.

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Chondrocyte Transduced with IRAP gene Inhibit IL-1  
Effects in OA Cartilage Cultures

To examine whether transplantation of chondrocytes transduced with the IRAP gene is chondroprotective, cartilage cultures were incubated in the presence or absence of exogenous IL-1 ( 500 units/ml) for 10 days and proteoglycan degradation monitored. On an average, 11.7 % of the total proteoglycan was degraded from IL-1 stimulated cultures transduced with the IRAP gene and 23.3 % from those transduced with the *lacZ* gene; whereas, proteoglycan degradation in control cultures (transduced with IRAP or *LacZ* gene, but not stimulated with IL-1) was 11.2 %. Thus, transplantation of chondrocyte with the IRAP gene inhibited IL-1-induced cartilage degradation significantly (ANOVA:  $F=12.5$ ,  $P<0.0001$ ,  $N=34$ ). The IL-1ra is a soluble protein that can bind to type I and type II IL-1 receptors, but is unable to mediate signal transduction through these receptors.

These experiments indicate that transduced chondrocyte are able to adhere to the surface of articular OA cartilage. Further, the transduced cells are able to express, process and secrete a biologically active cytokine, and this cytokine has biological

-28-

effects in the context of the microenvironment present across the surface of OA cartilage organ cultures.

The key to the problem of osteoarthritis is perhaps an approach that allows cartilage to heal.

5 Strategies aimed at developing chondroprotective agents face the challenge of delivering these agents to cartilage, an avascular tissue. Gene therapy used in the context of drug delivery appears to circumvent many of the problems associated with treating arthritis. Thus,

10 both viral and non-viral systems are being perfected to target genes to joints (6). Already, in vitro and in vivo experiments using adeno- and retroviral vectors have demonstrated gene delivery to synovium /synoviocytes (7-11). However, there are no reports of

15 gene transfer to chondrocyte or cartilage. The present invention demonstrates gene delivery to chondrocyte in culture using adenoviral vectors. The finding that greater than 90 % of chondrocyte transduced with the ntlacZ marker gene exhibited blue staining nuclei,

20 demonstrates the effectiveness of adenoviral mediated transduction and transgenic protein expression in these cells.

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In order to demonstrate gene delivery to articular cartilage, the target tissue, transplantation techniques were used to transfer transduced chondrocytes onto the surface of OA cartilage in culture. In situ staining for  $\beta$ -galactosidase and electron microscopy revealed that transduced chondrocytes adhered to cartilage, remained viable, and continued to express the transgenic protein even after 8 days in culture ( Fig 2A, B). Furthermore, it appears that transplanted chondrocytes had synthesized matrix components and thereby were integrating into the tissue (Figs 2F, G). Transplanted heterologous or autologous chondrocytes have been evaluated in animals as potential treatments for focal articular defects(24-26). Recently, chondrocyte transplantation has been successfully used to treat human patients with cartilage defects in their knees (13). However, this treatment was applied to more or less solitary lesions in an otherwise relatively normal joint. Cartilage degradation in arthritic joints is an end result of catabolic processes outstripping anabolic processes (5-6); therefore, it is desirable for chondrocyte transplantation alone to be sufficient to heal arthritic cartilage. However, it may be first desirable to inhibit the degradative processes before embarking on cartilage repair. Thus, in the present study, a combination of chondrocyte transplantation and

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gene delivery techniques are used to deliver IRAP gene to OA articular cartilage.

IRAP is reported to be a potentially therapeutic protein able to counteract the effects of IL-1, a cytokine recognized to function as a central mediator of inflammation and tissue destruction in arthritis. The data presented here indicate that chondrocyte transduced with IRAP cDNA upon transplantation adhere to cartilage, and express quantities of biologically active IRAP that is sufficient to counteract IL-1 induced proteoglycan degradation from OA cartilage organ cultures over a period of 10 days. This is a noteworthy achievement in that it has not been possible to demonstrate chondroprotection by delivery of IRAP by traditional routes of delivery (27, 28) or by transfer of IRAP gene to synovium (10, 11). The present invention demonstrates that transplantation of transduced chondrocytes is a viable strategy that may lead to treatment of osteoarthritis and other disorder of joints.



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Figure Legends

Figure 1. Expression of transgenic *LacZ* by human chondrocyte in culture. As described above, chondrocytes infected with Ad.RSVnt*LacZ* were fixed in 2% paraformaldehyde, reacted with 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal), and counterstained with neutral red. Cells expressing the transgene exhibited blue staining nuclei (A) and nuclei of control cells (mock-infected) did not stain (not shown). Immunohistochemical staining of transduced cells (blue nuclei) for expression of type II (B) or type I collagen (C). Red staining material is collagen.

Figure 2. Expression of transgenic *LacZ* by human chondrocyte transplanted onto cartilage organ cultures. Cells transfected with Ad.RSVnt*LacZ* vector and examined 2 (A) and 8 days (B) after transplantation onto cartilage organ cultures exhibited blue staining nuclei. Nuclei of cells subjected to mock-transfection did not stain (C). Chondrocytes transfected with Ad.CMV*LacZ* vector and transplanted onto the surface of intact cartilage (with the underlying subchondral bone) exhibited blue cytoplasmic staining (D); whereas control cartilage (with mock-transfected cells) exhibited no blue staining cells (E). Scanning electron micrograph

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(x 0.94 kx) of cartilage showing transplanted chondrocyte embedded in their territorial matrix (F). Higher magnification (G x 3.79kx) of transplanted chondrocytes showing the irregular surface and cytoplasmic processes of chondrocytes transplanted onto surface of cartilage cultures. Note the bundles of mature collagen fibers surrounding the cells.

Figure 3. Expression of IRAP by transduced chondrocytes transplanted on the cartilage surface. Cartilage cultures containing chondrocytes transduced with *LacZ* or IRAP gene incubated in the presence or absence of 500 units of IL-1 $\alpha$  for 10 days. The conditioned medium collected from these cultures was analyzed for IRAP content by ELISA. Means ( $\pm$  SE) not significantly different share superscripts (ANOVA: F= 56.7, N=16, P< 0.0001).

Figure 4. Transplantation of chondrocytes transduced with IRAP gene protects cartilage from IL-1-induced proteoglycan degradation. Cartilage cultures containing chondrocyte transduced with *LacZ* or IRAP gene incubated in the presence or absence of 500 units of IL-1 $\alpha$  for 10 days. The conditioned medium and cartilage from these cultures was analyzed for proteoglycan content using 1,9 dimethylmethylene blue dye-binding

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assay. Proteoglycan loss measured as percentage of proteoglycan released from cartilage into the medium during the experimental period. Means ( $\pm$  SE) not significantly different share superscripts (ANOVA: F= 5 12.5, N= 34,  $P < 0.0001$ ). There was no difference between controls transduced with *LacZ* and IRAP gene, and thus, these values were pooled.

## FIGURE 5. Generation of Ad.RSVntLacZ

The RSV promoter and a modified B-gal gene  
10 cloned into the proviral plasmid pADBglII to obtain pAdRSVntlacZ (nt=sequences that direct transgenic protein to the nuclear membrane).

Linearized pAdRSVntlacZ (NheI digestion) and truncated Ad5 derivative sub360 (ClaI digested to remove  
15 the E1A and E1B genes) DNA are co-transfected into 293 cells.

As a result of homologous recombination (within 9 to 16  $\mu$  of the adenoviral genome), a recombinant adenovirus containing the RSV promoter and  
20 the lacZ gene is produced. pEHX-L3 are plasmid backbone sequences. X represents a partial E3 deletion present in the sub360 derivative.

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## FIGURE 6. Generation of Ad.CMVLacZ

The CMV promoter and the E.Coli B-gal gene cloned into the proviral plasmid pADBglII to obtain pAdCMVLacZ.

5                   Linearized pAdCMVLacZ (NheI digestion) and truncated Ad5 derivative sub360 (ClaI digested to remove the E1A and E1B genes) DNA are co-transfected into 293 cells.

10                   As a result of homologous recombination (within 9 to 16 mu of the adenoviral genome), a recombinant adenovirus containing the CMV promoter and the lacZ gene is produced. pEHX-L3 are plasmid backbone sequences. X represents a partial E3 deletion present in the sub360 derivative.

## 15                   FIGURE 7. Generation of Ad.RSVhIL-1ra

The RSV promoter and the cDNA for human IL-1ra are cloned into the proviral plasmid pADBglII to obtain pAdRSVhIL-1ra.

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Linearized pAdRSVhIL-1ra (NheI digestion) and truncated Ad5 derivative sub360 (ClaI digested to remove the E1A and E1B genes) DNA are co-transfected into 293 cells.

5                   As a result of homologous recombination (within 9 to 16 mu of the adenoviral genome), a recombinant adenovirus containing the RSV promoter and the human IL-1ra cDNA is produced. pEHX-L3 are plasmid backbone sequences. X represents a partial E3 deletion  
10 present in the sub360 derivative.

#### Abbreviations

GEY's = A balance of salt solution of inorganic salts and D-glucose (See Amer. J. of Cancer (1936 27:55)).

15                   HAM F-12 = A commercially available nutrient medium mixture of inorganic salts, amino acids, vitamins, D-glucose, hypoxanthine, linoleic acid, lipoic acid, phenol red, putrescine HCL, sodium pyruvate, and thymidine.

20                   HEPES     =     (N-[2-hydroxyethyl)piperazine-N'-[2-ethanesulfonic acid]); Buffer (Sigma Chemical, St. Louis, Mo.

TESTICULAR HYALURONIDASE = enzyme from Worthington Biochemicals Corp., Freehold, N. J.

COLLAGENASE = enzyme from Worthington Biochemicals Corp.

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PIPES = piperazine-N,N"-bis[2-ethanesulfonic acid];  
buffer from Sigma Chemical

NP-40 = nonyl phenoxy polyethoxy ethanol; detergent from  
Sigma Chemical

5 EDTA = Ethylenediaminetetraacetic acid.

ANOVA = One way Analysis of Variance.

DMEM = Dulbecco's Modified Eagle Media, a commercially  
available medium containing inorganic salts, D-glucose,  
phenol red, sodium pyruvate, amino acids and vitamins.

10 Hanks Balanced Salt Solutions is a mixture of inorganic  
salts, D-glucose and phenol red (See Proc. Soc. Exp.  
Biol. Med. (1949) 71, 196, Modification - NIH).

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## References

1. Steinberg, J. J., and C.B. Sledge. 1991. Chondrocyte mediated cartilage degradation: regulation by prostaglandin E<sub>2</sub>, cyclic AMP and interferon  $\alpha$ . J. Rheumatol. (suppl 27) 18: 63-65.  
5
2. Dodge G. R., and A. R. Poole. 1989. Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal, rheumatoid, and osteoarthritic articular cartilages and in explants of bovine articular cartilage culture with interleukin1. J. Clin. Invest. 83: 647-661.  
10
3. Fell, H. B., and R. W. Jubb 1977. The effect of synovial tissue on the breakdown of articular cartilage in organ culture. Arthritis Rheum. 20: 1359-1371.
4. Hamerman, D. 1969. Cartilage changes in the rheumatoid joint. Clin. Orthop. Rel. Res. 64:91-97.  
15
5. Bora F. W. Jr., and G. Miller. 1987. Joint physiology, cartilage metabolism, and the etiology of osteoarthritis. Hand Clinics 3; 325-336.
6. Bland, J.H., and S.M. Cooper. 1984. Osteoarthritis: a review of the cell biology involved and evidence for reversibility. Management rationally related to known genesis and pathophysiology. Semin. Arthritis Rheum. 14: 106-132.  
20  
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-38-

7. Bandra, G., P.D. Robbins, H.I. Georgescu, G.M. Mueller, J.C. Glorioso, and C.H. Evans.1992. Gene transfer to synoviocytes: prospects for gene treatment of arthritis. DNA Cell Biol. 11:227-231.
- 5 8. Bandra, G., G.M. Mueller, J. Galea-Lauri, M.H. Tindal, H.I. Georgescu, M.K. Sucharek, G.L. Hung, J.C. Glorioso, P.D. Robbins, and C.H. Evans.1993. Intraarticular expression of the interleukin-1 receptor antagonist protein by ex vivo gene transfer. Proc. Natl. Acad. Sci. USA 90:10764-10768.
- 10 9. Roessler, B.J., E.D. Allen, J.M. Wilson, J.W. Hartman, and B.L. Davidson. 1993. Adenoviral-mediated gene transfer to rabbit synovium in vivo. J. Clin. Invest. 92: 1085-1092.
- 15 10. Roessler, B.J., J.W. Hartman, D.K. Vallance, J.M. Latta, S.L. Janich, and B.L. Davidson. 1995. Inhibition of interleukin-1 induced effects in synoviocytes transduced with the human IL-1 receptor antagonist cDNA using an adenoviral vector. Hum.Gene Ther. 6: 307-315.
- 20 11. Hung, G.L., J. Galea-Lauri, G.M. Mueller, H.I.Georgescu, L.A. Larkin, M.K. Sucharek, M.H. Tindal, P.D. Robbins, and C.H. Evans. 1994. Suppression of intra-articular responses to interleukin-1 by transfer of the interleukin-1 receptor antagonist gene to synovium. Gene Ther.1: 64-69.
- 25 12. Evans, C.H., and P.D. Robbins.1994. Gene therapy for arthritis. In: Gene Therapeutics: Methods and Applications of Direct Gene Transfer Wolff, J. A. (ed.).pp 320-343, Birkhauser: Boston, MA.
- 30



13. Brittberg, M., A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, L. Peterson. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. 331: 889-95.
14. Dinarello, C. A. 1989. Interleukin-1 and its biologically related cytokines. Adv. Immunol. 44: 153-205.
15. Arend, W.P, and J-M. Dayer. 1990. Cytokines and cytosine inhibitors or antagonists in rheumatoid arthritis. Arthritis Rheum. 33: 305-315.
16. Smith, R.J., N.A. Rohloff, L.M. Sam, J.M. Justen, M.R. Diebel, and J.C. Cornette. 1989. Recombinant human interleukin-1 $\alpha$  and recombinant human interleukin-1 $\beta$  stimulate cartilage matrix degradation and inhibit glycosaminoglycan synthesis. Inflammation. 13: 367-382.
17. Kumkumian, G. K. et al. 1989. Platelet-derived growth factor and IL-1 interactions in rheumatoid arthritis. Regulation of synoviocyte proliferation, prostaglandin production, and collagenase transcription. J. Immunol. 143:833-837.
18. Gilman, S. C. 1987. Activation of rabbit articular chondrocyte by recombinant human cytokines. J. Rheumatol. 14:1002-1007.
19. Dayer, J.M. et al. 1986. Human recombinant interleukin 1 stimulate collagenase and prostaglandin production by human synovial cells. J. Clin. Invest. 77:645-648.

-40-

20. Arend, W.P. 1993. Interleukin -1 receptor antagonist. Adv. Immunol 54:167-227.
21. Hurwitz, D.R., and G. Chinnadurai. 1985. Evidence that a second tumor antigen coded by adenovirus early gene region E1a is required for efficient cell transformation. Proc. Natl. Acad. Sci. USA 82:163-167.
22. Walsh, C., and C. L. Cepko. 1988. Clonally related cortical cells show several migrations patterns. Science (Wash., DC). 241: 1342-1345.
- 10 23. Baragi, V.M., H. Jordan, and R.R. Renkiewicz. 1992. A protocol for rapid screening of proteoglycan-degrading metalloproteinase inhibitors. J. Pharm. Method. 27(2):1-5.
- 15 24. Chesterman, P.J., and A.U. Smith. 1968. Homotransplantation of articular cartilage and isolated chondrocyte: an experimental study in rabbits. J. Bone Joint Surg. Br. 50:184-97.
- 20 25. Green, W.T., Jr. 1977. Articular cartilage repair: behavior of rabbit chondrocyte during tissue culture and subsequent allografting. Clin. Orthop. 124:237-50.
26. Aston, J.E., and G. Bentley. 1986. Repair of articular surfaces by allografts of articular and growth-plate cartilage. J. Bone Joint Surg. Br. 68:29-35.

-41-

27. Wooley, P. H., J. D., Whalen, D. L., Chapman, A. E., Berger, K. A., Richard, D. G., Aspar et.al., 1993. The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice. Arthritis Rheum. 36: 1305-1314.

28. Lewthwaite, J. C., S. M. Blake, T. E., Hardingham, and B. Henderson. 1993. The effect of interleukin-1 receptor antagonist on the progression of antigen-induced arthritis in the rabbit. Trans. Orthop. Res. Soc. 18: 237.

While the forms of the invention herein constitute presently preferred embodiments, many others are possible. It is not intended herein to mention all of the possible equivalent forms or ramifications of the invention. It is understood that the terms used herein are merely descriptive rather than limiting and that various changes may be made without departing from the spirit or scope of the invention.

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What Is Claimed Is:

1. Genetically modified chondrocytes comprising articular chondrocytes genetically modified with at least one gene which encodes for a protein having a therapeutic or prophylactic activity for an arthritic condition.  
5
2. The chondrocytes of Claim 1, wherein the gene that has been inserted into the chondrocytes encodes for a gene product selected from the group consisting of a cytokine (excepting IL-1), an anticytokine, an anti-adhesion molecule, an expression vector for ribozyme targeted against cytokine or matrix metalloproteinase (MMP), an anti-oxidant, an antibody, an antisense RNA, an MMP inhibitor and a cartilage growth factor.  
10  
15
3. The chondrocytes of Claim 2, wherein the inserted gene is a cytokine antagonist.
4. The chondrocytes of Claim 3, wherein the inserted gene is an interleukin-1 receptor antagonist.
5. Transfected chondrocytes comprising recombinant chondrocytes comprising articular chondrocytes recombinantly modified with a gene which encodes for a protein that has a therapeutic or prophylactic activity for an arthritic condition.  
20
6. The chondrocytes of Claim 5, wherein the gene that has been inserted into the chondrocytes encodes for a gene product selected from the group consisting of a cytokine (excepting IL-1), an  
25

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anticytokine, an anti-adhesion molecule, an expression vector for ribozyme targeted against cytokine or matrix metalloproteinase (MMP), an anti-oxidant, an antibody, an antisense RNA, an MMP inhibitor and a cartilage growth factor.

7. The chondrocytes of Claim 5, wherein the inserted gene is an anti-cytokine.

8. The chondrocytes of Claim 5, wherein the inserted gene is an interleukin-1 receptor antagonist.

9. A method of treating an arthritic condition comprising:

subjecting a patient in need thereof of to an effective amount of recombinant chondrocytes comprising articular chondrocytes genetically modified with at least one gene which has a therapeutic or prophylactic activity for an arthritic condition.

10. The chondrocytes of Claim 9, wherein the gene that has been inserted into the chondrocytes encodes for a gene product selected from the group consisting of a cytokine (excepting IL-1), an anticytokine, an anti-adhesion molecule, an expression vector for ribozyme targeted against cytokine or matrix metalloproteinase (MMP), an anti-oxidant, an antibody, an antisense RNA, an MMP inhibitor and a cartilage growth factor.

11. The chondrocytes of Claim 9, wherein the inserted gene is an anti-cytokine.

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12. The chondrocytes of Claim 9, wherein the inserted gene is an interleukin-1 receptor antagonist.

13. A method of delivering a nucleic acid to cartilage comprising the steps of:

5. providing chondrocytes modified with a gene which has a therapeutic or prophylactic activity for an arthritic condition, and

delivering the modified chondrocytes to a mammal having a diseased cartilaginous state.

10 14. The chondrocytes of Claim 13, wherein the gene that has been inserted into the chondrocytes encodes for a gene product selected from the group consisting of a cytokine (excepting IL-1), an anticytokine, an anti-adhesion molecule, an expression  
15 vector for ribozyme targeted against cytokine or matrix metalloproteinase (MMP), an anti-oxidant, an antibody, an antisense RNA, an MMP inhibitor and a cartilage growth factor.

20 15. The chondrocytes of Claim 13, wherein the inserted gene is an anti-cytokine.

16. The chondrocytes of Claim 13, wherein the inserted gene is an interleukin-1 receptor antagonist.

25 17. The method of Claim 13, wherein the modification technique utilized is an ex vivo technique utilizing autologous chondrocytes.

18. The method of Claim 13, wherein the modification technique is an ex vivo technique utilizing allogenic chondrocytes.

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19. The method Claim 13, wherein multiple genes are inserted into chondrocytes.

20. The method of Claim 19, wherein the genes are interleukin-1 receptor antagonist and a cartilage growth factor.

21. The method of Claim 13, further comprising:

employing recombinant techniques to produce a viral vector containing the gene;

transfecting the viral vector into a viral packaging cell line for the production of a particle capable of expressing the gene; and

infecting the chondrocytes using the viral particle obtained from the viral packaging cell line.

22. The method of Claim 13, wherein the chondrocytes are modified by a viral vector.

23. The method of Claim 13, wherein the chondrocytes are modified by a non-viral method selected from the group consisting of liposome, calcium phosphate, electroporation and DEAE-Dextran.

24. The method of Claim 13, comprising delivering the gene to the chondrocytes utilizing an adenovirus.

25. The method of Claim 24, comprising delivering the gene to the chondrocytes utilizing an adeno-associated virus.

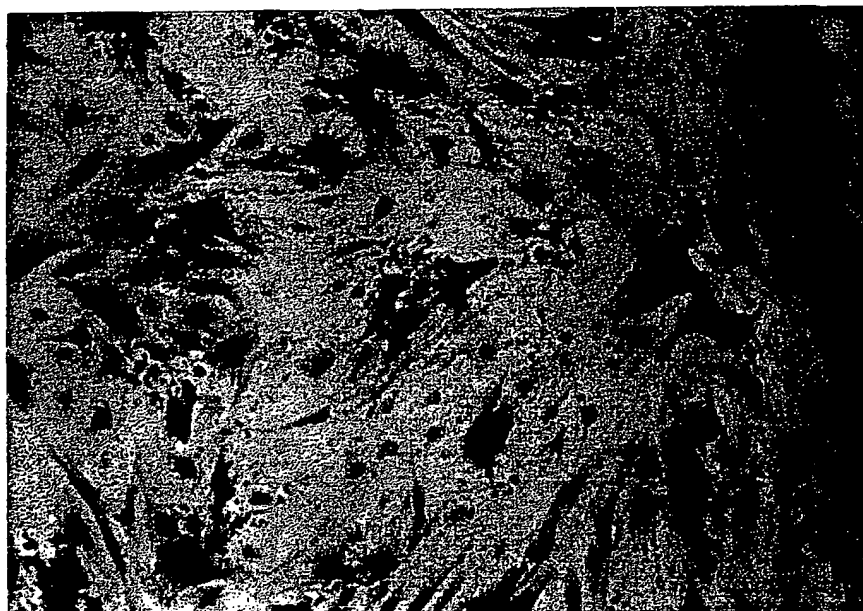
-46-

26. The method of Claim 24, comprising delivering the gene to the chondrocytes utilizing an  $\alpha$  virus.

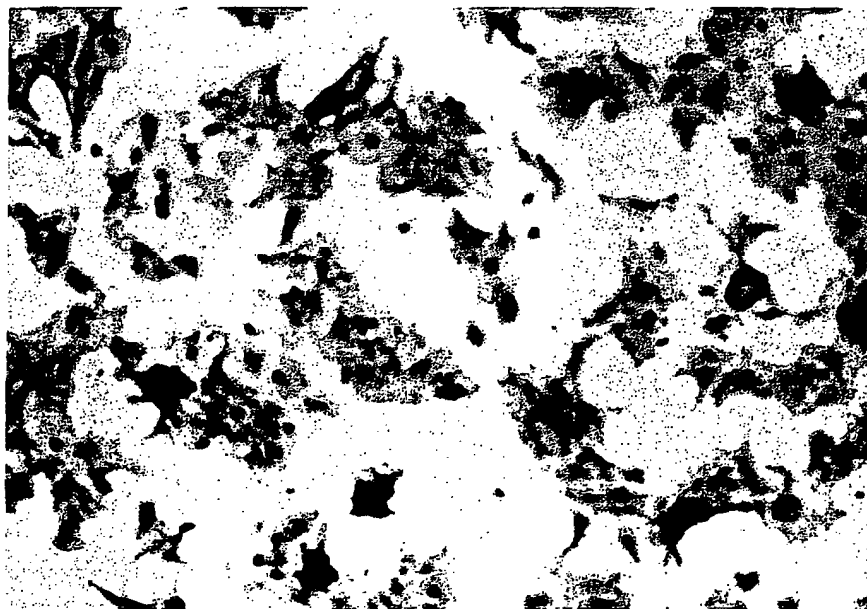
5 27. The method of Claim 24, comprising delivering the gene to the chondrocytes utilizing a herpes virus.



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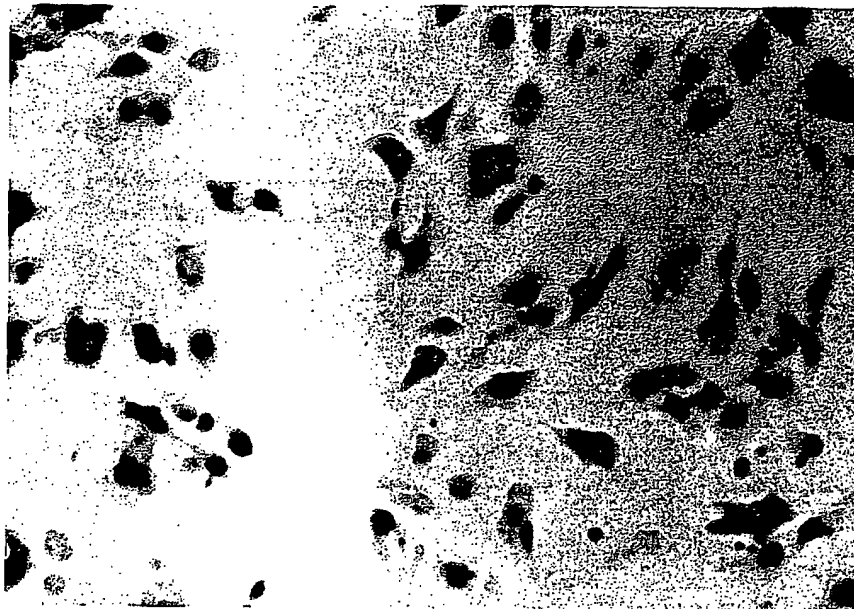


**FIG - 1A**

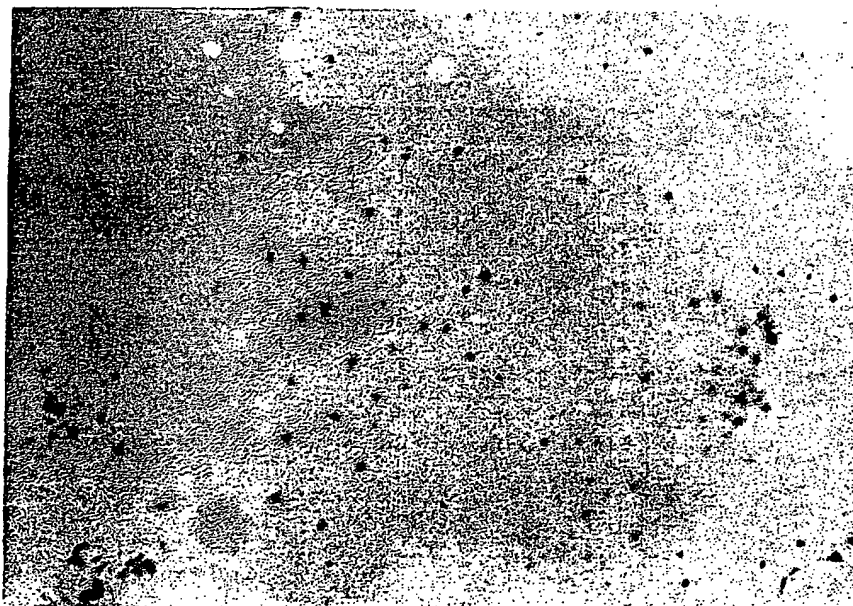


**FIG - 1B**

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**FIG - 1C**



**FIG - 2A**

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**FIG - 2B**



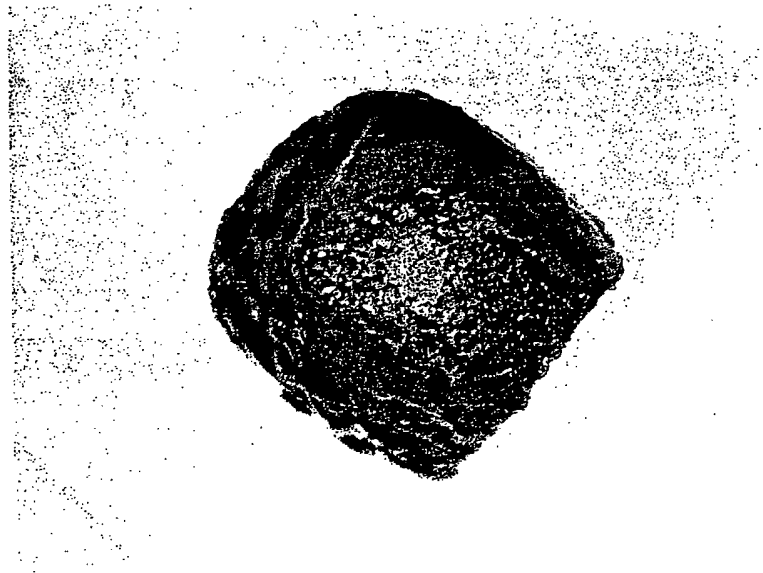
**FIG - 2C**

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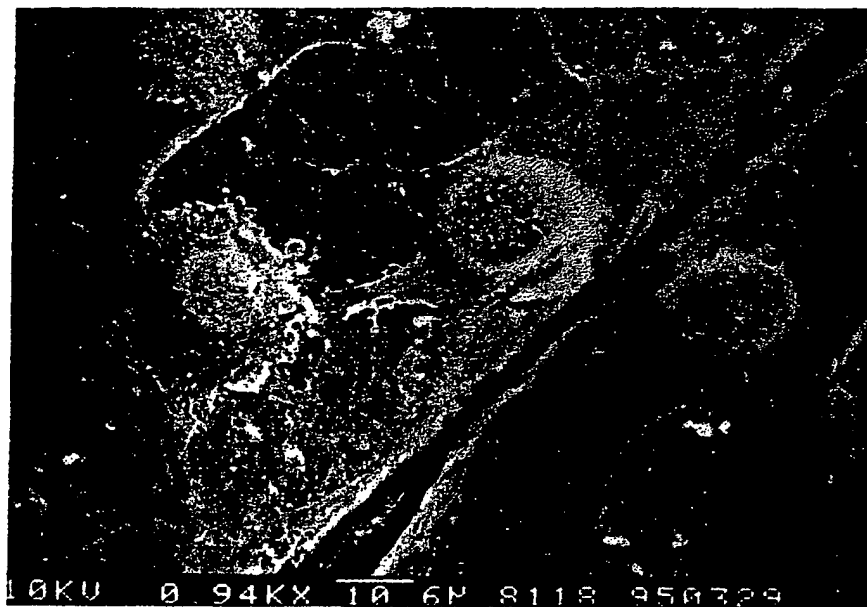
**FIG - 2D**



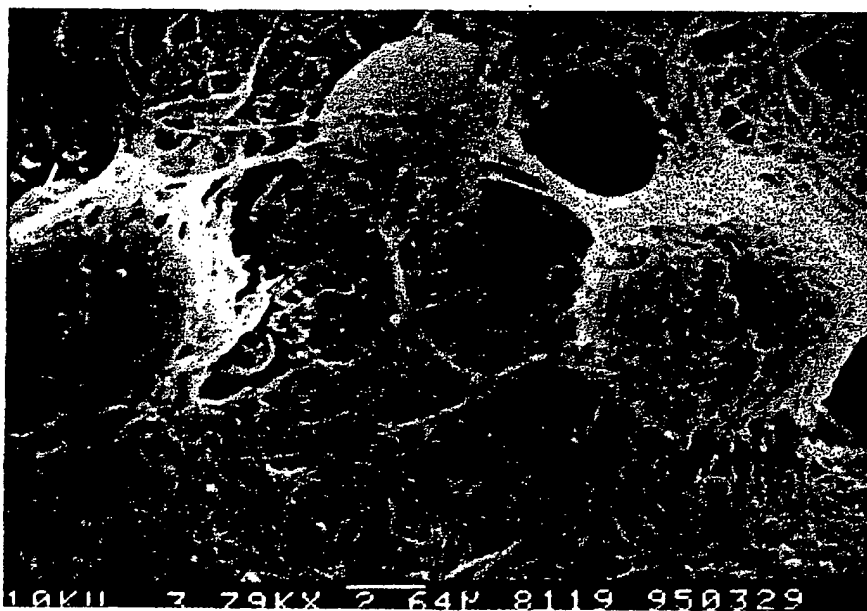
**FIG - 2E**

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**FIG - 2F**

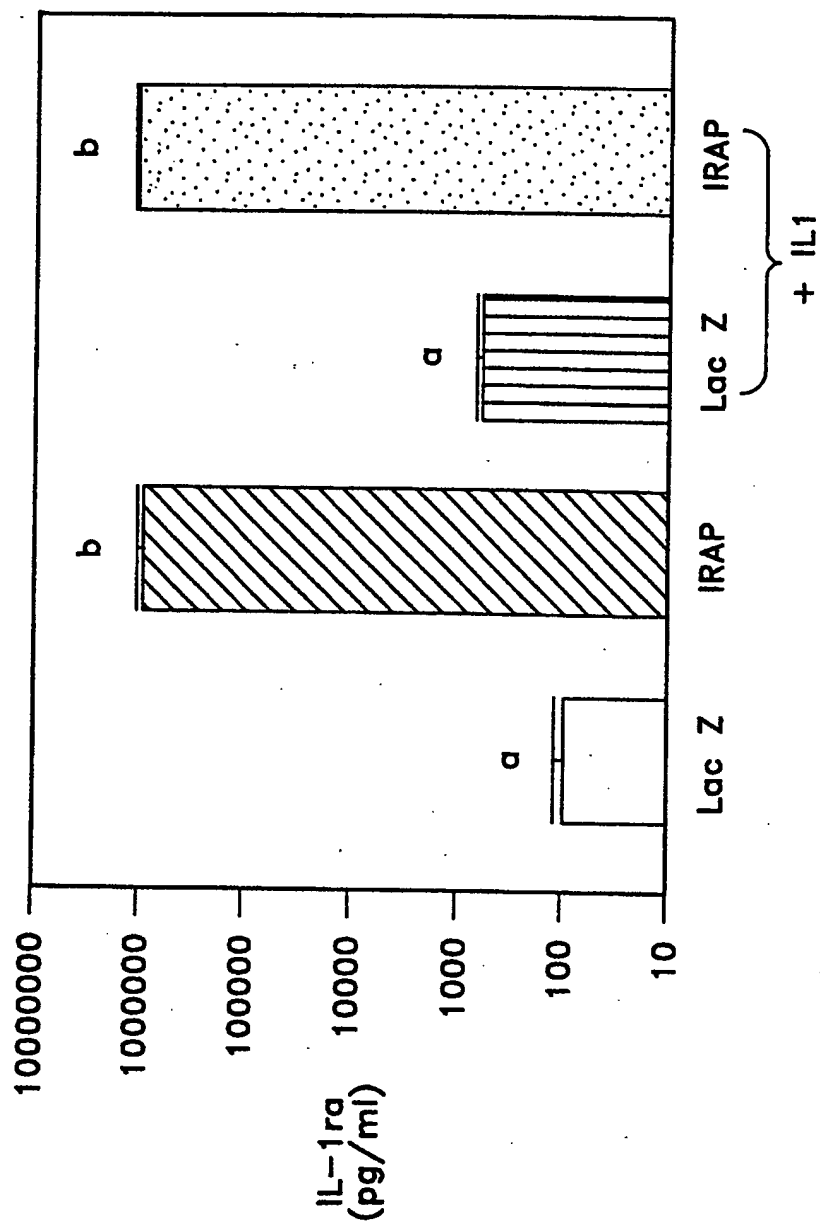


**FIG - 2G**

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FIG-3



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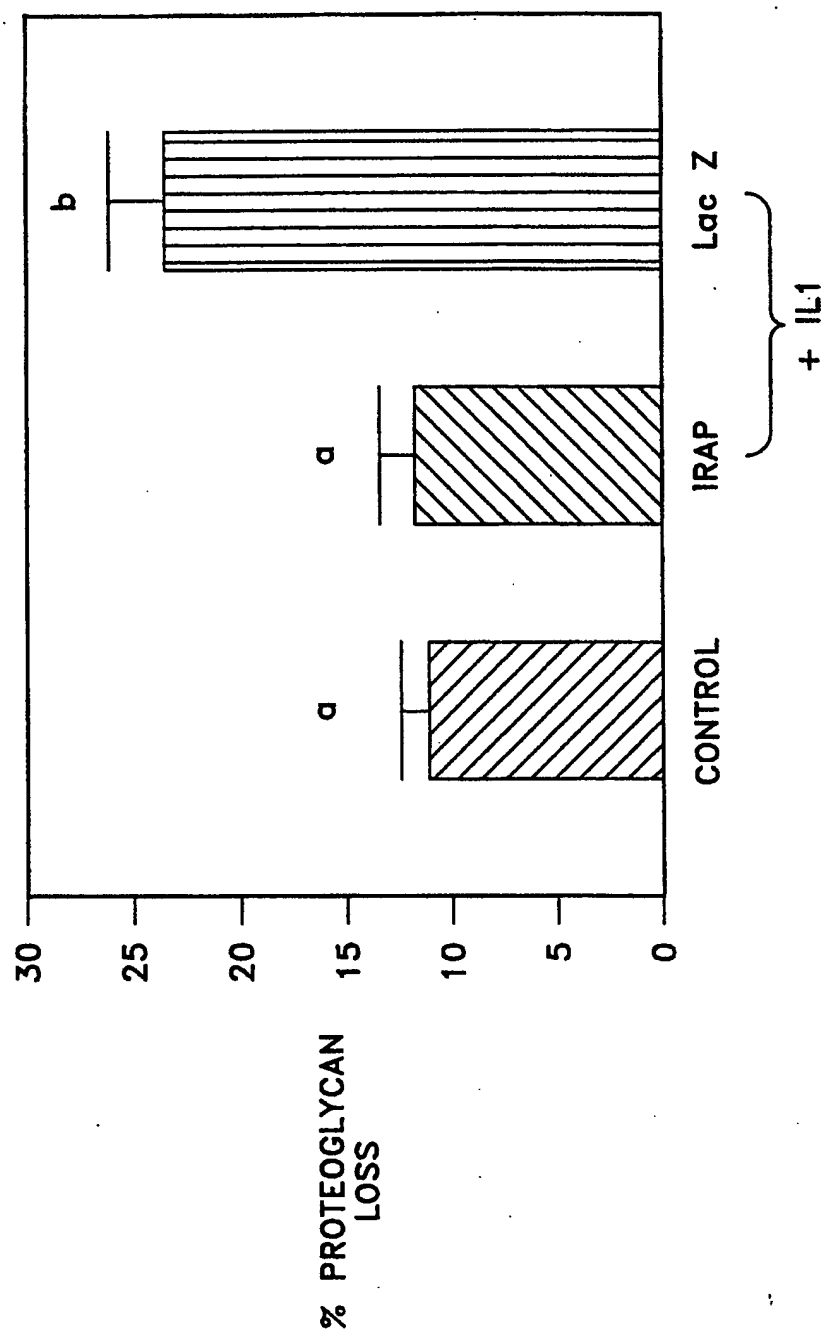
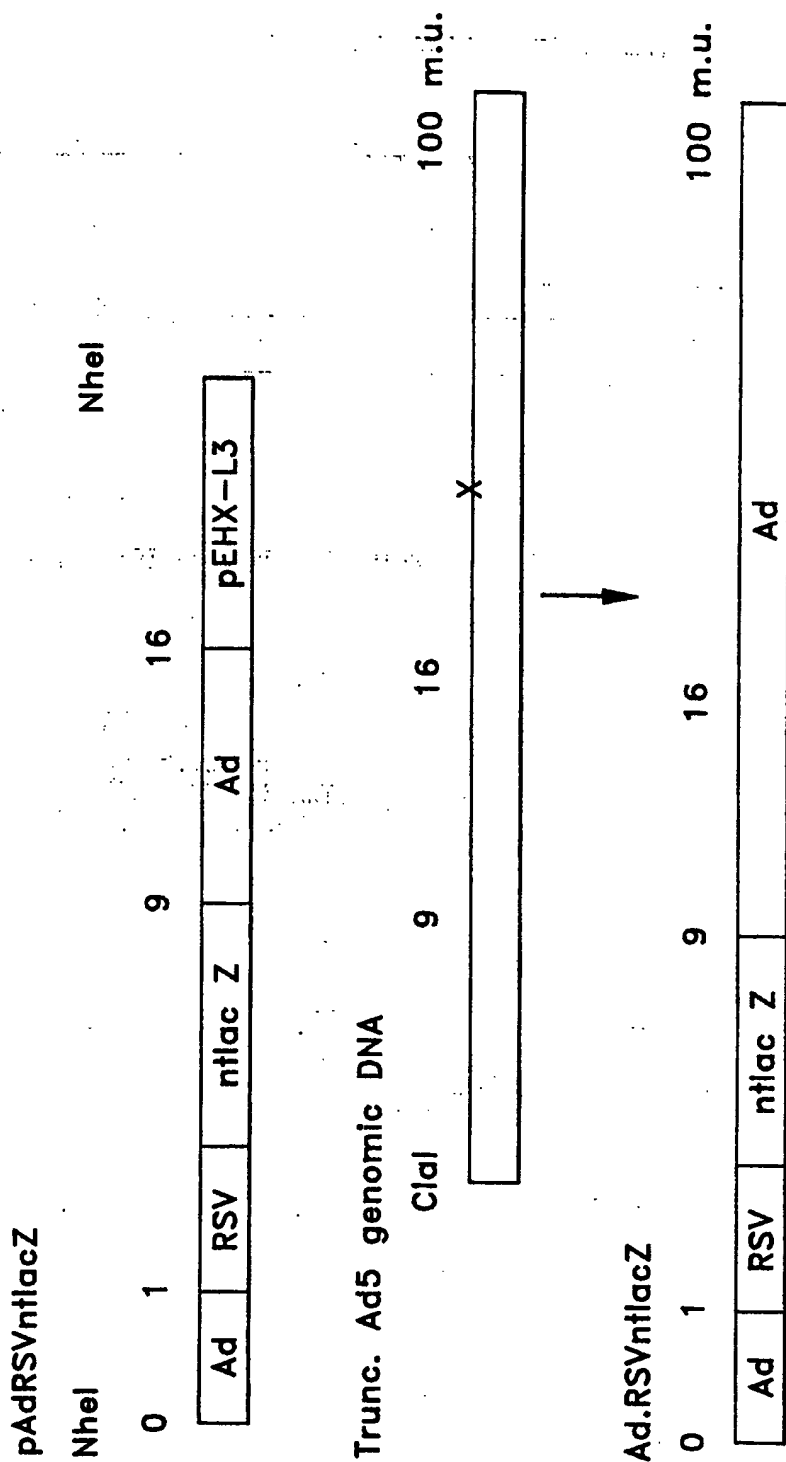


FIG-4

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FIG-5





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# FIG-6

pAdCMVlacZ

NheI

0 1 9 16



Trunc. Ad5 genomic DNA

Clal

9

16

100 m.u.



Ad.CMVlacZ

0 1 9 16

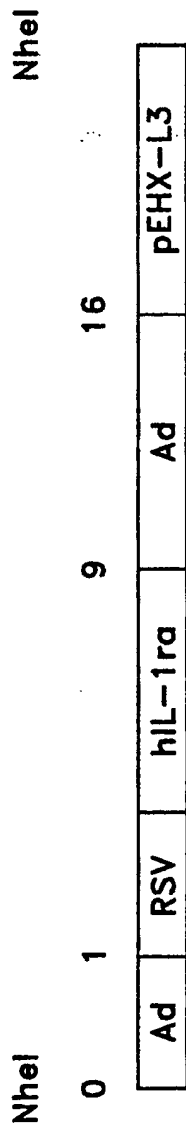
100 m.u.



10/10

# FIG-7

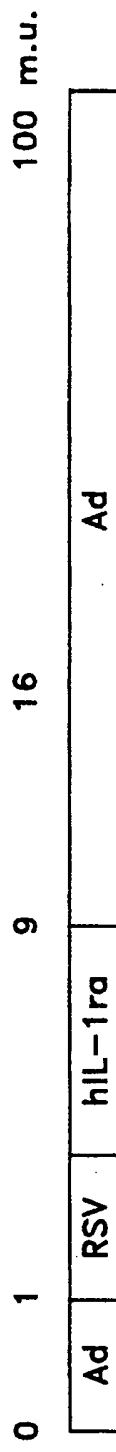
pAdRSVhIL-1ra



Trunc. Ad5 genomic DNA



Ad.RSVhIL-1ra



## INTERNATIONAL SEARCH REPORT

International Application No

PC, US 96/04902

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12N15/12 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE,A,42 19 626 (WEHLING PETER PRIV DOZ DR MED) 23 December 1993 cited in the application see the whole document ---	1-27
X	WO,A,94 09118 (GEN HOSPITAL CORP.;GOLDRING MARY (US)) 28 April 1994 see page 3, line 5 - page 5, line 5 ---	1-27
P,X	THE JOURNAL OF CLINICAL INVESTIGATION, vol. 96, no. 5, November 1995, pages 2454-2460, XP000577051 BARAGI ET AL: "TRANSPLANTION OF TRANSDUCED CHONDROCYTES PROTECTS ARTICULAR CARTILAGE FROM INTERLEUKIN 1-INDUCED EXTRACELLULAR MATRIX DEGRADATION" see the whole document ---	1-27
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

25 July 1996

Date of mailing of the international search report

31. 07. 96

Name and mailing address of the ISA

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Authorized officer

Sitch, W

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/04902

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,P, X	ARTHRITIS AND RHEUMATISM, vol. 38, no. 9, September 1995, page S401 XP002009407 VALLANCE ET AL: "VARIABLE TRANSGENIC EXPRESSION OF TISSUE INHIBITOR OF METALLOPROTEASES-1 BY SYNOVIOCYTES AND CHONDROCYTES IN VITRO" see abstract 1487 -----	1,2,5,6, 9,10,13, 14, 17-19, 21-27

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/04902

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9, 13, 17-27  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body (Article 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/ composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PC17US 96/04902

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-4219626	23-12-93	NONE	
WO-A-9409118	28-04-94	NONE	